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P01/7700 0.00-0212522.7

2. Patent application number

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0212522.7

3. Full name, address and postcode of the or of each applicant (underline all surnames)Shimadzu Research Laboratory (Europe) Ltd
Wharfside
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Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

Incorporated in UK

7309507001

4. Title of the invention

Selective Binding and Analysis of
Macromolecules

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom
to which all correspondence should be sent
(including the postcode)McNeight & Lawrence
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0001115001

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McNeight & Lawrence 31/05/02

12. Name and daytime telephone number of person to contact in the United Kingdom

Dr Ian R Lambert 0161 480 6394

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SELECTIVE BINDING AND ANALYSIS OF MACROMOLECULES

5 This invention relates to selective binding and analysis of macromolecules with particular, but by means exclusive, reference to quantitative analysis of proteins or protein function in complex mixtures.

10 Since the completion of the first eukaryote *-Saccharomyces cerevisiae-*, genomic sequence databases of entire organisms have become easily available. So far, the enormous collection of data has been mostly utilised to facilitate the identification of the proteins expressed in the cell. The entire repertoire of proteins synthesised in a cell is also known as *proteome*.

15 In a typical proteomics experiment, isolation of the proteins from the rest of cell extract is followed by fractionation of the proteome so that less complex protein mixtures may be obtained using two dimensional gel electrophoresis (2D-GE) and/or chromatographic methods. Thereafter, the resulting protein mixtures are digested using a proteolytic enzyme (trypsin), generating peptide mixtures which are subsequently analysed by mass spectrometry. Protein characterisation relies on the use of soft ionisation techniques such as matrix assisted laser desorption ionisation (MALDI) mass spectrometry (MS) and electrospray ionisation mass spectrometry (ESI-MS).

25 From the genomic sequences it is possible to generate all theoretical proteins expressed at any time (*virtual proteome*). However, mass spectrometric analysis (either using peptide mass finger (PMF) or using tandem mass spectrometry in conjunction with collision induced dissociation (CID)) reveals which gene products are actually being synthesised by a cell under a given set of physiological conditions (*real proteome*). Crucial to the functional analysis of biological systems is the ability to quantify alterations in protein abundance resulting from internal or external perturbations of the cell. In recent

years, many efforts have been devoted to develop chemical strategies for quantitative analysis of proteins in complex mixtures.

The common strategy involves derivatisation of each set of proteins/
 5 with isotopic variants of the same chemical reagent/label. The samples are then cor-
 and differentiated using mass spectrometry after purification. This approach is base
 the assumption that the two sets of isotopically labelled peptides behave ide-
 throughout sample manipulation and mass spectrometric analysis. Oda *et al* intr
 isotopic labelling during cell growth by allowing one cell state to grow in ^{15}N me
 10 another in ^{14}N nitrogen (Oda Y, Nagasu T, Chait BT, Nat Biotechnol 2001 Apr; 19(
 82). Comparison between the ion intensities of the peptide incorporating both i
 variants allows relative quantification between two cell states. The approach is ex
 and is not applicable to those types of proteins deriving from cells where introduc
 isotopic label is difficult. In addition, the mass difference between peptide ion
 15 causes variations in the level of ^{15}N incorporation due to peptide composition.
 methods requires an initial protein identification step prior to the quantification.

More recently, an alternative strategy based on the selective incorpor
 isotopically label amino acids has been proposed. Martinovic et al (S Martinov
 20 Vecnstra, GA Anderson, L Pasa-Tolic and RD Smith, J. Mass Spectrom. 2002 37:9
 described a method in which proteins were extracted from organisms grown in a ri
 medium or a minimal medium to which isotopically labelled leucine (Leu D_{10}) was
 The two protein extracts were mixed and analysed by capillary isoelectric focusing
 coupled with Fourier transform ion cyclotron resonance mass spectrometry (FTICR
 25 incorporation of the isotopically labelled species shows no effect on the CIEF sep
 efficiency of the proteins and both isotopically labelled and unlabelled version
 specific protein are observed in the same spectrum. The difference in the mas
 between the labelled and unlabelled is utilised to determine the number of Leu re
 present in a particular protein. A drawback associated with the technique is the hi

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of the instrumentation. Pratt and collaborators (JM Pratt, DHL Robertson, SJ Gaskell, I Riba-Garcia, SJ Hubbard, K Sidhu, SG Oliver, P Butler, A Hayes, J Petty, R Beynon, proteomics 2002, 2, 157-163) have reported the use of selective incorporation of leucine in conjunction with proteolytic digestion and MALDI analysis. In this approach, the utility of leucine labelling is reported as means of enhancing confidence in protein identification in peptide mass fingerprinting (PMF). Because leucine is an abundant amino acid in many genomes, most peptides will contain at least one leucine residue. Labelling in vivo with the two versions of Leu in each digest fragment renders possible identification of the exact number of amino acid residues within the molecule. The extra information can be exploited during an identification process which is conducted using a database searching engine to reduce the search space. In principle the method is amendable to quantification.

However, regardless of its sensitivity and its selectivity, mass spectrometry is still unable to cope with mixtures containing peptides derived from several proteins. The presence of many components generates chemical noise which is detrimental to detection of those ions whose chemical features are relatively poorly suited to the ionisation method employed. Prefractionation is applied in order to resolve a mixture into its components. Of chromatographic techniques, biotin-avidin affinity capture-release based methods followed by liquid chromatography are highly appealing due to the possibility of obviating the use of gel electrophoresis (which is known to be unsuitable in the analysis of low abundance proteins). Of particular relevance to the present application is the isotope coded affinity tag (ICAT) technique. In this approach, proteins extracted from cells can be labelled, separated and quantified (Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R, Nat Biotechnol 1999 Oct;17(10):994-9 and International Publication WO 00/11208), the contents of both of which are hereby incorporated by reference.

The label is composed of three parts: firstly, an affinity tag (typically biotin), which is utilised to isolate ICAT labelled peptides; secondly, a linker incorporating stable

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isotopes; and thirdly a reactive group with specificity towards thiol groups. The biotin group is selectively recognised during the affinity extract step by an avidin moiety attached to a chromatographic column.

5 The linker moiety typically contains either eight hydrogens (light) or deuteriums (heavy), and provides for relative quantification. The reactive group having specificity towards thiol groups enables the label to react with cysteine residues. The overall protocol involves derivatisation of all proteins present followed by digestion of the entire set and affinity extraction prior to tandem mass spectrometric analysis. Comparison
10 of the precursor ions of the two isotopically labelled ion species enables the relative abundance of one ion against another to be determined.

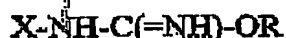
 However, there are a number of problems and disadvantages associated with the above described ICAT technique. Firstly, the abundance of cysteine amino acid
15 residues in proteins is relatively low. Secondly, the reactivity of the thiol specific group is not highly specific and, furthermore, side reactions can take place. Thirdly, the reproducibility of the reaction varies from an experiment to another. This hampers quantification, introducing a substantially unquantified variable into the quantitative analysis. Additionally, ionisation efficiency and fragmentation efficiency of the derivatised
20 peptides selected for tandem mass spectrometry can be altered by the introduction of the ICAT label. The combined effect of these experimental factors, together with factors such as differences in retention times for the two sets of isotopic variants displayed under chromatographic analysis, renders problematic the use of the ICAT procedure as a user friendly method.

25 The present invention overcomes the above named problems and disadvantages.

- 5 -

According to a first aspect of the invention there is provided a method for selectively binding macromolecules having a lysine functionality comprising the steps of:
providing a sample containing one or more species of macromolecules, each having a lysine functionality;

5 providing a binding reagent having the formula



or



10

where X is an affinity label that selectively binds to a capture reagent, R is a residue group, and L is a linker moiety;

introducing the binding reagent to the sample so as to affect a guanidination reaction between the binding reagent and said one or more species of macromolecules, thereby producing one or more affinity containing homoarginine derivatives;

15 optionally modifying the affinity label containing homoarginine derivatives to produce further affinity label containing homoarginine derivatives; and capturing homoarginine derivatives using the capture reagent that selectively binds X.

20

According to a second aspect of the invention there is provided a method for analysing one or more proteins, protein functions and/or peptides in one or more samples comprising the steps of:

25 providing a binding reagent having the formula



or



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where X is an affinity label that selectively binds to a capture reagent, R is a residue group, and L is a linker moiety;

introducing the binding reagent to the one or more samples so as to effect a guanidination reaction between the binding reagent and proteins and/or peptides having a lysine functionality, thereby producing one or more affinity label containing homoarginine derivatives;

optionally modifying the affinity label containing homoarginine derivatives to produce further affinity label containing homoarginine derivatives;

capturing affinity label containing homoarginine derivatives using the capture reagent that selectively binds X;

and performing an analysis of affinity label containing homoarginine derivatives.

There are a number of advantages associated with this scheme. The method is specific to lysine amino acid residues, which are more abundant than the cysteine amino acids analysed in Gygi et al, *abid*. Unlike Gygi et al, the method is not subject to side reactions, since the ϵ -amino group of the isourea moiety is more specific than the sulphhydryl moiety of Gygi et al. To date, evidence about conversion of the N-terminus amino groups is very rare, with just a single paper reporting guanidination of the N-terminus when glycine is the N terminal amino acid residue. Furthermore, in comparison to Gygi et al, the binding reagents of the present method provide an increase in ionisation efficiency.

The advantages are also applicable to the first aspect of the invention.

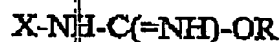
The step of modifying the homoarginine derivatives may comprise converting proteins present into peptides. This conversion might be performed enzymatically or chemically. Alternatively, it is possible to analyse the direct product of the guanidination

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reaction between the binding reagent and a protein "directly", i.e. without digestion or fragmentation of the protein into peptides (top-down approach).

The protein functions and/or peptides may be identified by the analysis of the affinity label containing homoarginine derivatives. The analysis may comprise the step of comparing data generated by an analytical technique with sequence databases. Typically, this is performed using tandem mass spectrometry (MS/MS). It is also possible to interpret data directly.

Relative expression levels of proteins in two or more samples containing proteins may be determined in a method comprising the steps of:
providing a series of binding reagents having the formula



or



where X is an affinity label that selectively binds to a capture reagent, R is a residue group, and L is a linker moiety, and wherein the chemical formulae of the binding reagents in the series are identical but each binding reagent in the series comprises a different combination of isotopes so that the binding reagents in the series are isotopically labelled by way of the molecular mass of each binding reagent in the series being different to the molecular masses of the other binding reagents in the series;

introducing a different binding reagent from the series to each sample so as to effect, in each sample, a guanidination reaction between a binding reagent and proteins having a lysine functionality, thereby producing a plurality of isotopically labelled, affinity label containing homoarginine derivatives;

combining the samples;

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optionally converting proteins present in the affinity label containing homoarginine derivatives into peptides;

capturing affinity label containing homoarginine derivatives using the capture reagent that selectively binds X; and

5 performing an analysis of affinity label containing homoarginine derivatives in which the relative abundances of a subset of homoarginine derivatives which differ only by virtue of their isotopic labelling are measured, thereby determining the relative expression levels of the protein from which the subset of homoarginine derivatives originated.

10

In conjunction with the determination of the relative expression levels of the proteins, the proteins, protein functions and/or peptides may be identified by the analysis of the affinity label containing homoarginine derivatives. The analysis may comprise the step of comparing data generated by an analytical technique with sequence data. It is also
15 possible to interpret data directly.

20

Advantageously, analysis comprises mass spectrometric analysis. Other techniques, such as electron microscopy, might be contemplated. Mass spectrometric analysis may comprise tandem mass spectrometry. This is especially useful in conjunction with peptide/protein sequencing (denovo or database assisted), for identifying proteins, protein function and peptides. One stage of mass spectrometry is conveniently utilised to determine the relative abundances of a subset of homoarginine derivatives which differ only by virtue of their isotopic labelling, through comparison of ion peaks in a mass spectrum associated with each homoarginine derivative in the subset.

25

The method may further comprise the step of releasing captured affinity label containing homoarginine derivatives from the capture reagent prior to the step of performing an analysis. The capture reagent may comprise part of a chromatographic separation system which separates chemically different affinity label containing

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homoarginine derivatives. Conveniently, the chromatographic separation system utilises liquid chromatography. However, other methods can be used, such as other chromatographic separation systems. The homoarginine derivatives might be separated out in situ through immobilisation on a chip or another solid surface or matrix, in which instance of step of releasing captured affinity label containing homoarginine derivatives is not mandatory.

Absolute quantification of the proteins and/or peptides may be obtained:

In the first and second aspects of the invention, R may be a moiety that is compatible with the guanidination reaction. R may be CH_3 , C_2H_5 or C_3H_7 .

According to a third aspect of the invention there is provided a reagent for selectively binding macromolecules having a lysine functionality having the formula



or



where X is an affinity label that selectively binds to a capture reagent, R is a residue group, and L is a linker moiety. X may be biotin or a modified biotin. R may be a moiety that is compatible with the guanidination reaction. R may be CH_3 , C_2H_5 or C_3H_7 .

Methods and reagents in accordance with the invention will now be described with reference to the accompanying drawings in which:-

Figure 1

shows the guanidination of lysine into homoarginine;

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Figure 2

shows the guanidination of a lysine containing macromolecule into a homoarginine derivative using a binding reagent;

Figure 3

shows a scheme for quantifying differential protein expression;

Figure 4

shows an alternative form of a binding reagent;

Figure 5

shows a reaction scheme for preparing a binding reagent;

Figure 6

shows a LC/ES-MS analysis of lysine terminal standard peptides treated with O-methylisourea. Selected ion monitoring (SIM) of four marker-ions is performed selecting the singly and doubly charged ions of lysine (K) and its corresponding homoarginine terminal peptide (K*), (a) 35 pmole of interleukin $[M+2H]^{2+}$ and $[M+H]^+$ at 503.2 and 1005.4 are indicated with 2+ and 1+ respectively. (b) 50 pmole of AFLDASK $[M+2HF]^{2+}$ and $[M+H]^+$ at 376.25 and 751.25 are indicated with 2+ and 1+ respectively. The arrows indicate the expected retention times of the unmodified lysine containing peptides;

Figure 7

shows a LC/ES-MS analysis of an equimolar mixture of interleukin (K, 12.5pmole) with its guanidinated counterpart (K*,12.5pmole) acquired in selected ion monitoring. Doubly charged ion signals are indicated

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with +2 while singly charged ion signals correspond to +1, (b) SIM LC/ES-MS analysis of a equimolar mixture of 20 pmole of AFLDASK with AFLDASK*;

Figure 8

shows a LC/ES-MS analysis of a solution containing equal amount (15 pmole) of ALFDASK, AFLDASR and the homoarginine terminal analogue AFLDASK*. The lysine terminal peptide is eluted first followed by the arginine terminal counterpart. SIM analysis of all six ions indicates that the homoarginine terminal peptide ions are the dominant signals in the MS chromatogram regardless of the charge state selected; and

Figure 9

shows an isocratic separation of a solution containing equal amount (31.5pmole) of AFLDASK, AFLDASR and the AFLDASK*. The conditions employed correspond to the concentration of mobile phase (0.05% (v/v) TFA and 32.6% (v/v) acetonitrile) at which the peptides were eluted in gradient mode.

The present invention exploits the guanidination reaction to *inter alia* provide improvements to the technique of Gygi *et al* and WO 00/11208.

Figure 1 depicts the guanidination of lysine, typically at around pH 10 or greater, into homoarginine using O-methyl isourea. Figure 2 depicts a modified scheme in accordance with the invention in which guanidination of a macromolecule 1 having a lysine functionality is performed using an isourea derivative 2, producing a homoarginine derivative 3. The macromolecule 1 can be, for example, a peptide or a protein. The

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isourea derivative 2 is a binding reagent provided by the present invention in which X is an affinity label that selectively binds to a capture reagent. It is possible to utilise an isourea derivative 3 in place of isourea derivative 2, in which L is a linker moiety and X has the meaning described in relation to derivative 2.

5 In a general sense, the invention provides methods and reagents for selectively binding and capturing macromolecules having a lysine functionality. Once bound and captured a macromolecule can be analysed by a suitable technique such as mass spectrometry or a spectroscopic method. In important embodiments the invention provides
10 analytical reagents and analytical methods for the isolation, purification and identification of proteins and peptides in mixtures of proteins and peptides. The method employs binding reagents which exploit the reactivity between lysine amino groups and isourea derivatives to isolate, purify and determine peptides and proteins present in a mixture. Homoarginine derivatives are created which isolate lysine containing proteins and
15 peptides.

Figure 3 shows a scheme for quantifying differential protein expression. Two protein mixtures 10, 12 which represent different cell states are treated with chemically identical, isotopically different binding reagents 14, 16. Thus, a first protein mixture 10
20 may be treated with an isotopically "light" reagent 14, and a second protein mixture 12 may be treated with an isotopically "heavy" reagent 16. Guandination produces a mixture of homoarginine derivatives 18, which, by virtue of the binding reagents utilised, is "light" and a mixture of homoarginine derivatives 20 which, by virtue of the binding reagents utilised, is "heavy". The mixtures 18, 20 are combined, and proteins (including those
25 forming part of the homoarginine derivatives) are digested to peptides using techniques which are well known in the art. The homoarginine derivatives (now comprising peptides) are isolated using a separation technique commensurate with the nature of the affinity label, such as a chromatographic technique. The isolated homoarginine derivatives are then separated and analysed using a chromatographic technique such as liquid

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chromatography and mass spectrometry (LC-MS). Micro / nano capillary liquid chromatography (μ LC) can be used.

Identical peptides emanating from the mixtures 10, 12 give rise to chemically identical homoarginine derivatives which more or less coelute from the LC. However, due to the "light" and "heavy" nature of the binding reagents, these homoarginine derivatives have different molecular masses which are identifiable in the mass spectrum at different values of mass-to-charge ratio. Thus, relative quantification of protein expression is possible by comparing the relative abundances of the ion peaks corresponding to the "light" and "heavy" homoarginine derivatives. MS/MS analysis of the elutants provides sequence information which enables identification of the protein through computer searching of the experimentally obtained sequence information against databases. Further details of suitable analysis schemes can be found in WO 00/11208 and Gygi *et al.* It is also possible to perform identification of proteins and peptides using MS/MS without determining relative protein expressions, in which instance isotopic variants of the binding reagents are not required.

Isotopic variants of a binding reagent can be prepared by incorporating different isotopes into the linker moiety, such as is generally described in WO 00/11208 and Gygi *et al.* One scheme involves deuteration of a hydrogen containing backbone. Additionally, or alternatively, it is possible to utilise ^{15}N at one or both of the N positions in the isourea moiety.

In place of chromatographic isolation and separation, it may be possible to utilise other capture techniques in order to separate the homoarginine derivatives from the original sample. For example, the affinity label might be attached to a solid surface or a matrix. In a related embodiment, the binding reagent may be linked to a solid support or to a matrix such as shown in Figure 4. In such a scheme, it is possible to move proteins present in the sample which do not react with the binding reagent in a washing procedure.

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After attachment to a solid support, proteins can be digested in situ with a proteolytic enzyme and analysed directly, such as by mass spectrometry. The reaction scheme is amenable to automation.

5 In further embodiments of the invention, calibrations are performed using samples containing known concentrations of proteins, in order to obtain a quantitative relationship between peptide signal obtained during analysis and the absolute amount of protein present in the sample.

10 Methods

The following synthetic path can be used to synthesise binding reagents.

15 Preparation of biotin-2,3,5,6-tetrafluorophenyl ester

The method is described at page 43 of WO 00/11208, synthesis number 2 displayed at page 83, using 2 as a residue.

20 Synthesis and preparation of 0.5M ^{14}N and ^{15}N O-methylisourea solution

The method described is a modification of a procedure by Burgoyne *et al* to synthesise a similar compound (Burgoyne, D L, J Org Chem. 2000, 65, 152-155). A solution of 60 mg (1mmol) ^{14}N (or ^{15}N) urea (Aldrich, Milwaukee, WI) in 95 μL (126 mg, 1mmol) of dimethyl sulfate (Aldrich, Milwaukee, WI) was heated and agitated at 50°C for 5 hours. 250 μL of acetone was added and the solution was stirred for 1 hour. 1 mL of diethylether (Aldrich, Milwaukee, WI) was then added and the resulting mixture was stirred. The top layer was discarded and the addition of diethylether was twice repeated

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to afford ~60mg (~100 μ L) of *O*-methylisourea hemisulfate salt (~0.5 mmol, 49% yield). The product was dissolved in 900 μ L of water to give a ~0.5M solution.

Reaction between biotin-2,3,5,6-tetrafluorophenyl ester and *O*-methylisourea

The reaction is shown in Figure 5. Biotin-2,3,5,6-Tetrafluorophenyl ester 4 is reacted with *O*-methylisourea 5 to produce a binding reagent 6 through reaction of the primary amine on 5 with the ester moiety on 4.

The biotin containing binding reagent 6 is conveniently used in conjunction with avidin affinity chromatographic techniques.

Example

During analysis of peptide mixtures, many factors influence electrospray response. Selective suppression of ion signals hampers quantification of all components observed in the spectrum. An investigation was performed into the effect of the C-terminal amino acid on electrospray ionisation (ESI) response of peptides. More specifically, the ionisation efficiency of lysine, arginine and homoarginine terminal peptides and the effect of limited modification in peptide structure on differences in ESI response were investigated.

Methods

Interleukin, VQGEESNDK (Sigma), AFLDASR and AFLDASK with purity >96% (New England Peptide Inc) were used in concentrations of 2-20 pmol/ μ L. All experiments were performed on a single quadrupole mass spectrometer LCMS-2010 (Shimadzu, Japan) fitted with an electrospray source. Source conditions were maintained constant during the analysis. Data was acquired in selected ion monitoring mode (SIM) for a total of twelve selected ions. Components were separated on a Phenomenex Luna

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C₁₈ column (2.0mm id x 50mm). In gradient mode, mobile phase composition was 0.05% TFA (v/v) for the aqueous phase and acetonitrile/water (9:1 v/v) incorporating 0.05% TFA (v/v) for the organic phase. LC10ADVp HPLC pumps (Shimadzu, Japan) were used to deliver solvent at a flow rate of 250µl/min.

Results

Completeness of guandination reaction.

10 In order to verify the absence of starting material, lysine containing peptides treated with methylisourea were loaded onto a C₁₈ column and subjected to liquid chromatography prior to ES analysis. The separation has two advantages: first, it removes the excess of *O*-methylisourea which interferes negatively with the ionisation process. Second, all components in the reaction mixture are resolved and eluted separately into the

15 mass spectrometer. Subsequent detection by ES/MS allows comparison and quantification of the relative intensities of lysine and its homoarginine terminal counterpart. Figure 6 illustrates LC/ES-MS analysis of lysine terminal standard peptides treated with methylisourea. Selected ion monitoring (SIM) on four specific marker-ions corresponding to the singly and doubly charged ions of lysine and homarginine terminal peptides was

20 used to monitor whether peaks in the total ion chromatogram contain lysine terminal peptides.

These results demonstrate that the guanidation procedure is quantitative.

25 LC-MS analysis of mixtures containing lysine and arginine terminal peptides.

The completeness of guandination allows preparation of homoarginine terminal peptide solutions with known concentrations. Binary mixtures containing the same quantity of lysine terminal peptide and its analogous homoarginine terminal

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counterpart were prepared and a comparison between the two ion signals is performed. Figure 7A shows an MS chromatogram of 20 pmole of interleukin with its guanidinated analogue during LC separation followed by ES. The conditions employed during the chromatographic separation (composition of mobile phases, gradient profile, etc) were kept identical to those used in the previous experiment (see Figure 6). Total ion chromatogram (TIC) traces of homoarginine terminal peptides (corresponding to the sum of the singly and doubly charged ion signals) have higher relative intensity than lysine terminal ones. The main contribution is due to the relative ES response of the doubly charged ion peaks. This observation is also made with an equimolar mixture of AFLDASK with its guanidinated counterpart AFLDASK* (Figure 7B).

LS-MS analysis of a mixture containing AFLDASK, AFLDASR and its homoarginine terminal counterpart AFLDASK*.

To investigate how minor variations in peptide structure may affect signal intensity, the arginine terminal analogue AFLDASR was added to the mixture. Figure 8 indicates the ion chromatograms of a solution containing 31.5 pmole each of AFLDASK, AFLDASR and the homoarginine terminal derivative AFLDASK* separated by LC prior to MS detection. The arginine terminal peptide displays an ion chromatogram with intensity higher than the corresponding AFLDASK in accordance with the higher basicity of the arginine. By comparing the two sets of peptide ions produced by AFLDASR and AFLDASK*, the latter one generally displays ion signals with higher intensity. Unlike the

lysine and arginine/ homoarginine terminal peptides (AFLDASK vs AFLDASR/AFLDASK*) where the difference in ionisation efficiency can be related to the higher stabilisation of the protonated peptide, in the case of the AFLDASK and AFLDASK* the difference in ES response cannot be simply related to the effect of the guanidino group as both are identical. The difference can be attributed to the increased level of proton solvation provided by the extra methylene group in the peptide backbone.

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Effect of isocratic separation on ESI response of AFLDASK, AFLDASR and AFLDASK* peptide ions.

5 The effect of co-elution of three peptides on their ESI response was studied. The mixture previously analysed (see Figure 8) was run isocratically. The mobile phase was prepared with the same composition used to elute AFLDASR, AFLDASK and AFLDASK* in the gradient mode. Hence, no separation is achieved and all three peptide elute simultaneously into the mass spectrometer. None of the ion signals in the MS chromatogram are suppressed compared with the ion signals observed in the LC-MS experiment (see Figure 10 8). The co-presence of three peptides different in gas/phase basicity shows no effect on their ESI response. Figure 9 displays MS chromatograms of AFLDASK, AFLDASR and AFLDASK* for singly and doubly charged protonated ions.

15 Conclusions

The completeness of the conversion of lysine into homoarginine enables quantitative evaluation of how C-terminus modification affects ESI response. Guanidation produces variation in either gas phase basicity (between lysine and homoarginine) or in peptide structure (between arginine and homoarginine) resulting in an enhanced signal 20 intensity for the guanidinated peptide. Co-elution and concomitant ionisation of all three peptides shows no effect on the relative ESI responses.

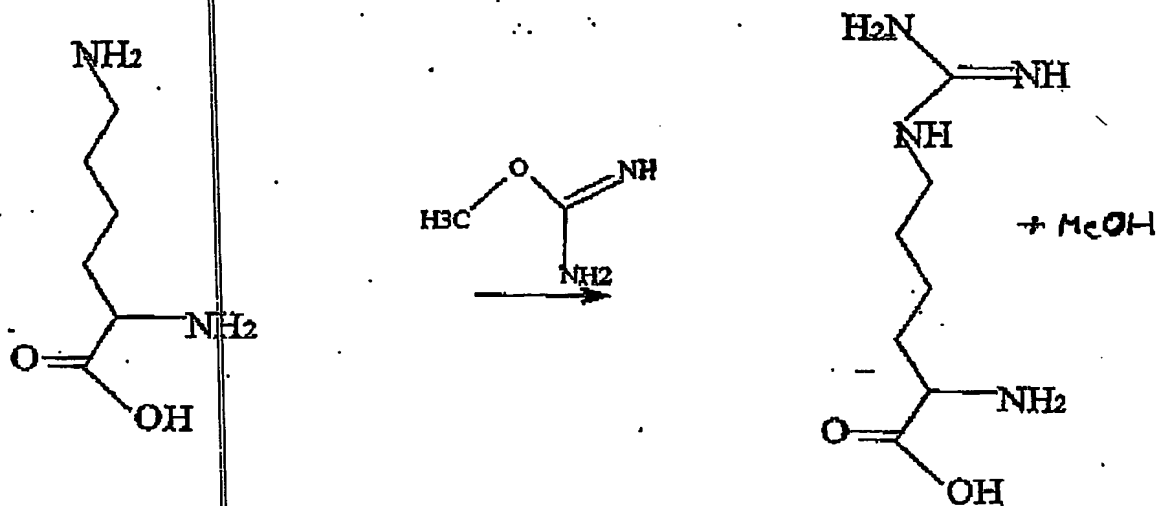


Fig. 1

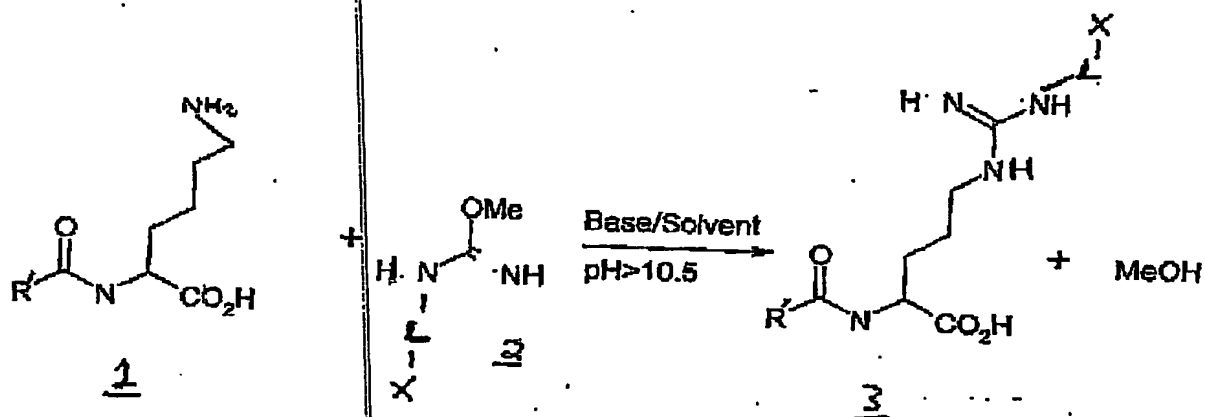
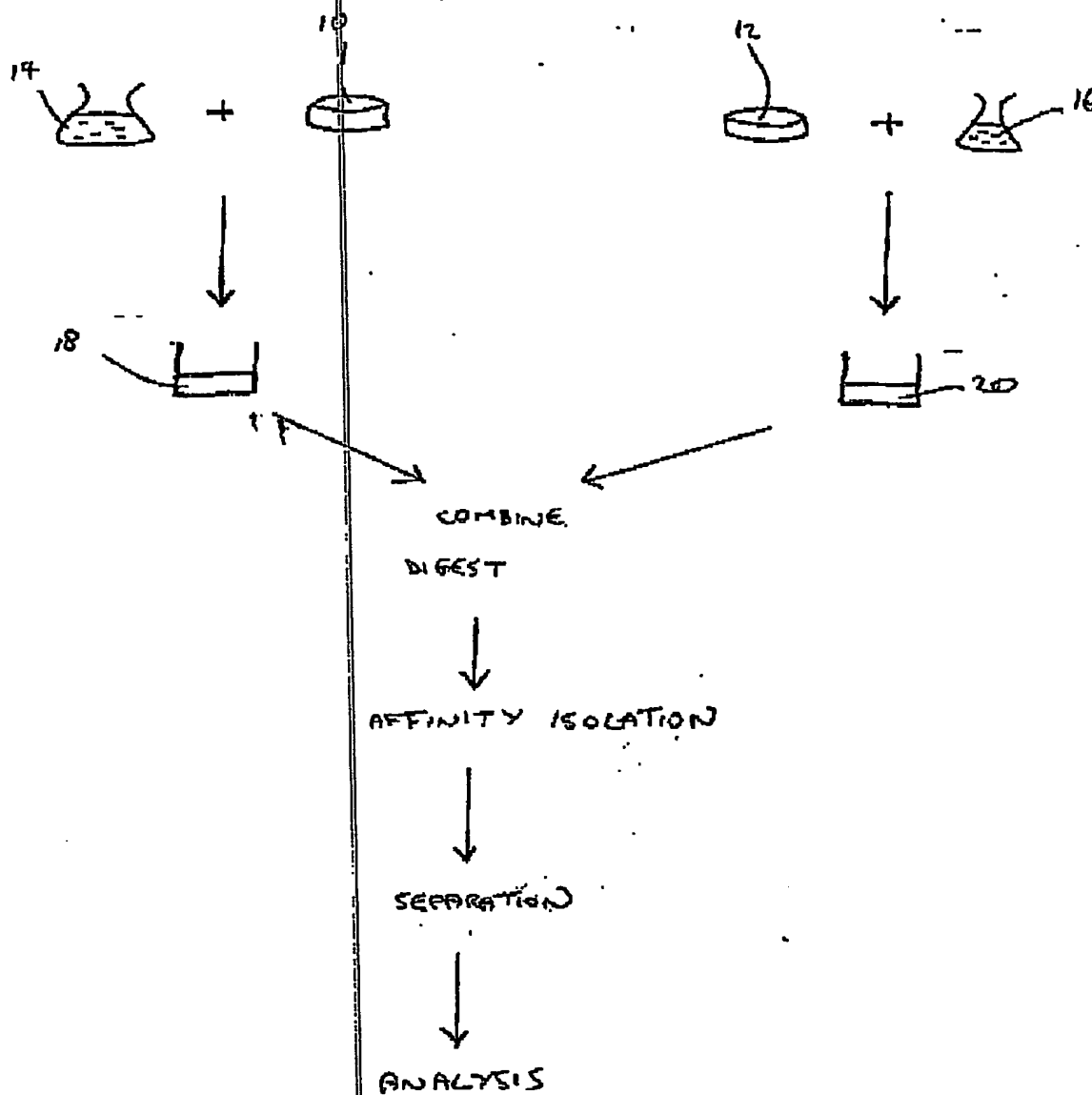


Fig. 2

Fig. 3

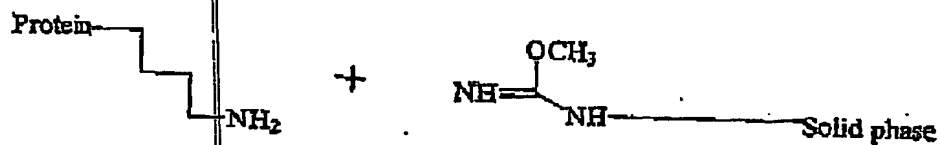


Fig. 4

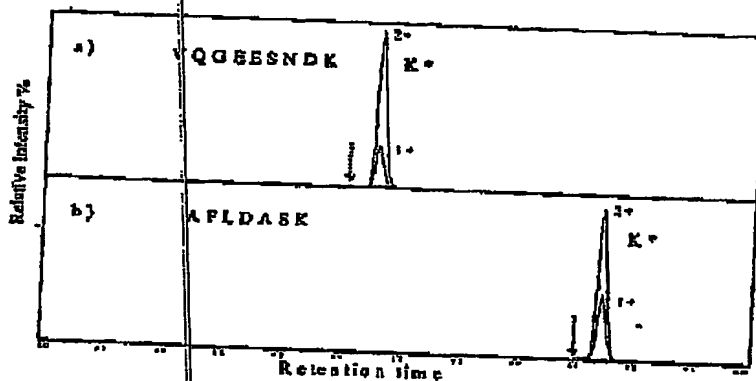
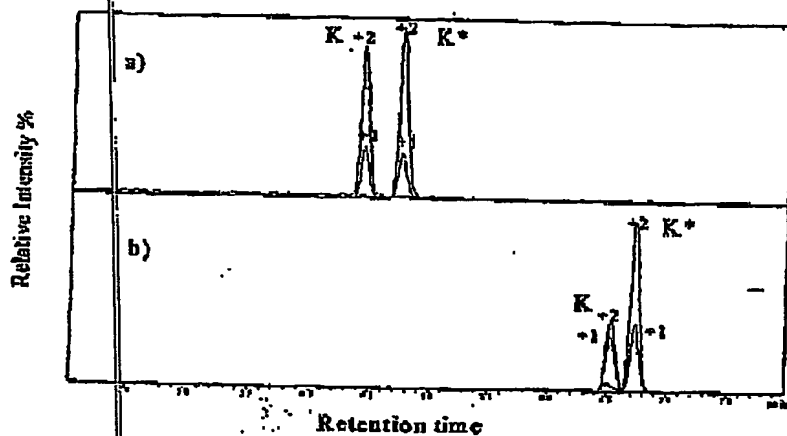
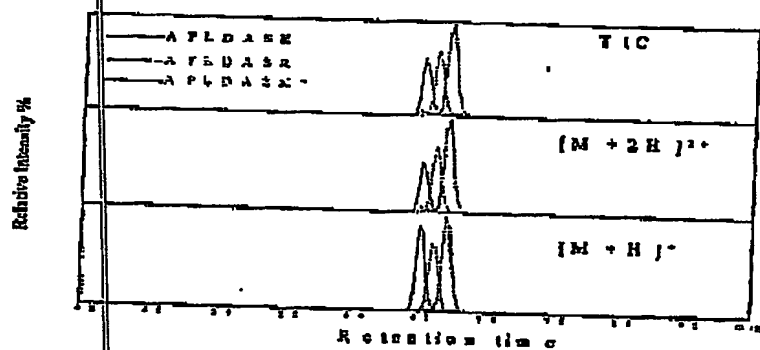


Fig. 5

Fig. 7Fig. 8

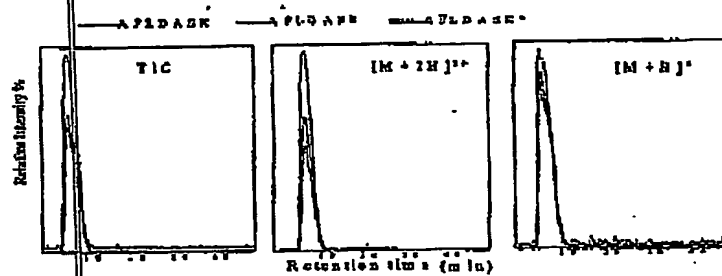


Fig. 9

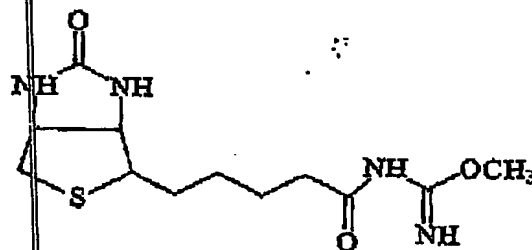
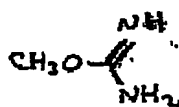
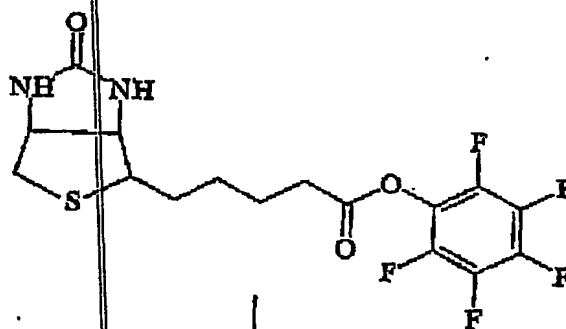


Fig. 5

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